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NATIONAL CANCER INSTITUTE
NATIONAL INSTITUTES OF HEALTH
DEPARTMENT OF HEALTH AND HUMAN SERVICES

■ IMMUNOLOGY

Genetic Engineering of Antibodies to Harness Their Clinical Potential

Kashmiri SVS, De Pascalis R, Gonzales NR, and Schlom J. SDR grafting—a new approach to antibody humanization. *Methods* 36: 25–34, 2005.

Dr. Syed V.S. Kashmiri of the NCI, Center for Cancer Research, Laboratory of Tumor Immunology and Biology, passed away on July 19, 2005. He will be remembered for his intellect, seminal and highly innovative contributions to the field of genetic engineering of antibody molecules, and for his extreme kindness. He will be very much missed.

Since the advent of hybridoma technology, a vast repertoire of murine monoclonal antibodies (mAbs) has been generated. The utility of these antibodies for the diagnosis and treatment of human cancers and infectious diseases is, however, limited—mainly because they elicit human anti-murine antibody (HAMA) responses in patients. To improve their clinical potential, murine antibodies have been genetically manipulated to replace their murine content with the amino acid residues present in their human counterparts, rendering them potentially less immunogenic in patients.

Initially, human-mouse chimeric antibodies were generated by replacing the murine-constant regions with those of the human antibodies. To further reduce the murine content, mAbs have been humanized by grafting their complementarity-determining regions (CDRs)—the segments of their variable regions that confer antigen-binding specificity and affinity on antibodies—onto the variable light and heavy frameworks of human immunoglobulin molecules. However, the xenogeneic CDRs of the humanized antibodies may still evoke an anti-idiotypic response in patients. To circumvent this, we have developed a new approach to humanize antibodies based on grafting only those residues of the xenogeneic CDRs that are critical in the antigen-antibody interaction onto the human antibody frameworks. An analysis of the three-dimensional structures of antibody-combining sites suggests that only 20% to 33% of CDR residues are critical in the antigen-antibody interaction. These residues, which are located in the regions of high variability and which are most likely to be unique to each antibody, are designated as specificity-determining residues (SDRs) (Padlan E et al. *FASEB J* 9: 133–9, 1995). SDR-grafted humanized antibody has a substantially reduced number of non-human residues as compared with those present in its CDR-grafted counterpart (Tamura M et al. *J Immunol* 164: 1432–41, 2000).

The first step in the humanization by SDR grafting is to choose the most appropriate human frameworks to be used as templates and to identify the framework residues, which are deemed essential for the antigen-binding properties of an antibody to be retained. The next step is to identify the SDRs. When the three-dimensional structure of the antigen-antibody complex (based on X-ray crystallographic studies) is known, the residues of the combining site directly involved in ligand contact can be easily identified. In the absence of a three-dimensional structure, potential SDRs could be identified

by examining the known crystal structures of antibody:ligand complexes that are currently available in the Protein Data Bank database. The database does not always lead to a definite conclusion about the dispensability of some SDRs, and may require experimental validation to assess the dispensability of the murine residues.

We have also used a more conservative approach to humanize an anti-CEA antibody. (CEA, or carcinoembryonic antigen, is overexpressed in a wide range of human carcinomas and is an excellent target for immunotherapy approaches.) This approach is based on grafting of the "abbreviated" CDRs—the stretches of CDR residues that include all the SDRs (De Pascalis R et al. *J Immunol* 169: 3076–84, 2002) (Figure 1). Since this approach does not require extensive genetic manipulation, it minimizes the risk of a loss in the antigen-binding of the antibody.

For a humanization protocol to achieve the desirable goal, it is important that the structural features of the target antibody are preserved. Humanization often results in a significant modification of the antigen-combining site structure and a consequent loss in the antigen-binding affinity of the antibody. To offset this loss, we have also used *in vitro* affinity maturation (De Pascalis R et al. *Clin Cancer Res* 9: 5521–31, 2003) to generate humanized antibodies with enhanced antigen-binding affinity and reduced immunogenic potential in human patients. It is anticipated that these novel recombinant technologies will result in the more effective use of monoclonal antibodies for the diagnosis and/or therapy of a range of human cancers.

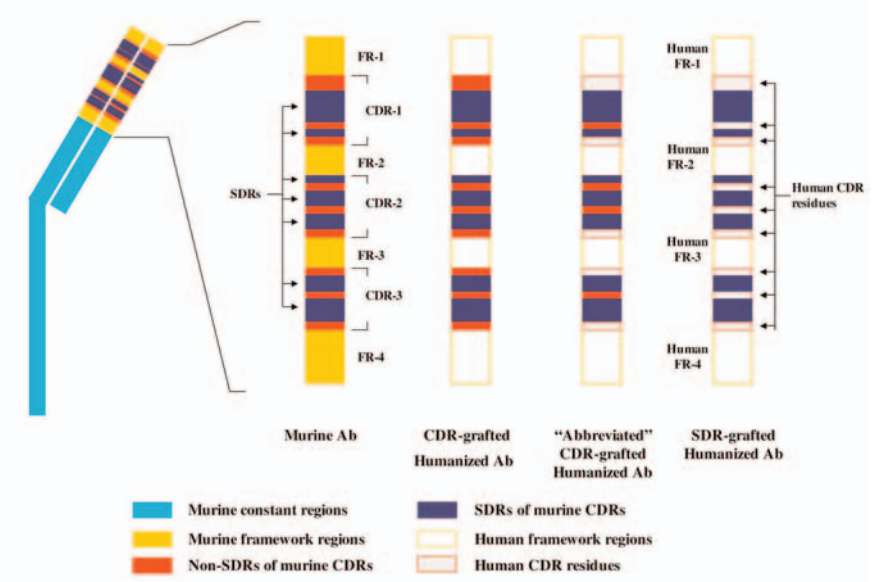

Figure 1. Schematic representation of the humanization protocols of the VL (variable light) region of an antibody, showing the VL region of a murine, complementarity-determining region (CDR)–grafted, "abbreviated" CDR-grafted, and specificity-determining residue (SDR)–grafted humanized antibody.

The NCI has filed seven patent applications to establish its intellectual property rights on the humanized antibodies generated in our laboratory and one to claim proprietary rights to the technique of humanizing antibodies by SDR grafting. Several pharmaceutical and biotechnology companies have already executed agreements and/or are in negotiation toward the development of the humanized antibodies we have generated.

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■ IMMUNOLOGY

A Novel Signaling Pathway Controlling Oxidative Stress-mediated Cell Death

Shen HM, Lin Y, Choksi S, Tran JH, Jin T, Chang L, Karin M, Zhang J, and Liu ZG. Essential roles of receptor-interacting protein and TRAF2 in oxidative stress-induced cell death. *Mol Cell Biol* 24: 5914–22, 2004.

Oxidative stress occurs when the amount of reactive oxygen species (ROS) in a system exceeds that system's ability to neutralize and eliminate them, for example, because of impaired antioxidant function. ROS, including superoxide anion, hydroxyl radicals, and hydrogen peroxide (H_2O_2), are known to be important in various biological and pathological processes (e.g., aging, inflammation, carcinogenesis) and in the pathogenesis of many human diseases (e.g., neural degenerative diseases, AIDS, cancer). One important aspect of the biological effects of ROS is their regulatory roles in cell death. ROS can act either as direct inducers or as signaling molecules in the cell death process triggered by many other stimuli, though the exact molecular targets of ROS and the signaling pathway controlling ROS-mediated cell death are largely elusive. Previous descriptions of ROS cytotoxicity are mainly based on the adverse effect of ROS on the cell membrane (membrane lipid peroxidation) or on mitochondria (both functionally and structurally). In our study, data provide novel evidence that ROS utilize some key

cell signaling molecules of tumor necrosis factor (TNF) signaling, such as RIP, TRAF2, or JNK, to control the life and death of the cell. Such findings will help to elucidate the importance of ROS and oxidative stress in various physiological and pathological conditions.

We found that H_2O_2 caused evident cell death in mouse embryonic fibroblast (MEF) cells in a caspase-independent manner. Interestingly, MEF cells in which the expression of *receptor interacting protein* (*RIP*) and *TNF receptor associated factor 2* (*TRAF2*) genes was knocked out are much more resistant to H_2O_2-induced cell death than the wild-type (wt) MEF cells. Moreover, the reconstitution of RIP and TRAF2 expression in their respective gene knockout cells significantly restored the sensitivity of the cells to H_2O_2, suggesting that RIP and TRAF2 are required for H_2O_2-induced cell death. RIP and TRAF2 are two key effector molecules in the TNF signaling pathway and mainly act as cell survival factors to protect against TNF-induced apoptosis via NF-κB activation. However, a recent report that RIP is required for death receptor–mediated caspase-independent cell death implies that RIP may also act as a transducer for cell death signals. Moreover, we found that H_2O_2-induced cell death is independent of TNF receptor 1 (TNFR1), as TNFR1$^{-/-}$ MEF cells were as sensitive as wt cells to H_2O_2-induced cell death. In addition, blockage of *de novo* protein synthesis failed to affect H_2O_2-induced cell death.

We also found that RIP and TRAF2 form a complex in response to H_2O_2 treatment. Using the co-immunoprecipitation technique, a rapid and transient interaction between RIP and TRAF2 was detected upon H_2O_2 exposure, and this interaction was independent of TNFR1 and TNFR1-associated death domain (TRADD), another critical molecule in the TNF signaling pathway. Recently, membrane lipid rafts have been implicated in cell signaling. We found that H_2O_2 rapidly induced the clustering of fluorescein isothiocyanate (FITC)–labeled cholera toxin B, a marker for membrane lipid rafts, and this was followed by RIP and lipid raft co-localization. Therefore, we believe that a RIP and TRAF2 interaction is an upstream event that initiates the cell death pathway, a process likely involving membrane lipid rafts and recruitment of some key signaling molecules. Lastly, we identified c-Jun N-terminal kinase (JNK) as the effector molecule in H_2O_2-induced cell death downstream of RIP and TRAF2. JNK is a member of the mitogen-activated protein kinase family that plays pivotal roles in cellular responses to oxidative stress–associated, caspase-independent cell death.

In summary, our study uncovers a novel signaling pathway regulating ROS (H_2O_2)–induced cell death, a process involving RIP, TRAF2, and JNK1. It appears that RIP and TRAF2 have critical functions in a much broader spectrum of signal transduction pathways than was originally thought and act as the convergence point to relay different stimuli or stressors to different downstream signaling pathways that determine the life and death of a cell.

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■ MOLECULAR BIOLOGY

A Promising Alternative Approach to Breast Cancer Therapy by Disrupting Estrogen Receptor Zinc Finger Function

Wang LH, Yang XY, Zhang X, Mihalic K, Fan YX, Xiao W, Howard OMZ, Appella E, Maynard AT, and Farrar WL. Suppression of breast cancer by chemical modulation of vulnerable zinc fingers in estrogen receptor. *Nat Med* 10: 40–7, 2004.

The estrogen receptor has long been recognized as a major molecular component in the progression and promotion of breast carcinoma. Once bound by estrogen, the receptor, acting as a transcription factor, can activate specific genes involved in cell cycle processes, cell survival, and cell metastasis. Endocrine therapy for breast cancer involves the administration of selective estrogen receptor modulators (SERMs), termed antiestrogens, which block the action of estrogens at the ligand level. However, the currently available antiestrogens possess mixed estrogenic and antiestrogenic activity, and the development of resistance is common. Secondly, estrogen receptors may be further activated by non-steroidal growth factors, such as epidermal growth factor (EGF), and other pathways. Thus, instead of targeting the receptor's activity at the ligand level, it may be advantageous to find new agents that block the growth of breast cancer cells by targeting the receptor's ability to function as a transcription factor.

The estrogen receptor is composed of discrete functional domains, including the hormone-binding, transactivation, and DNA-binding domains (DBDs). The DBD contains two non-equivalent Cys_4 zinc finger motifs that are essential to estrogen receptor–mediated transcription. X-ray crystallographic and nuclear magnetic resonance (NMR) studies have established that these dual zinc fingers function cooperatively in both estrogen receptor dimerization and DNA binding by stabilizing the secondary and tertiary structure of the

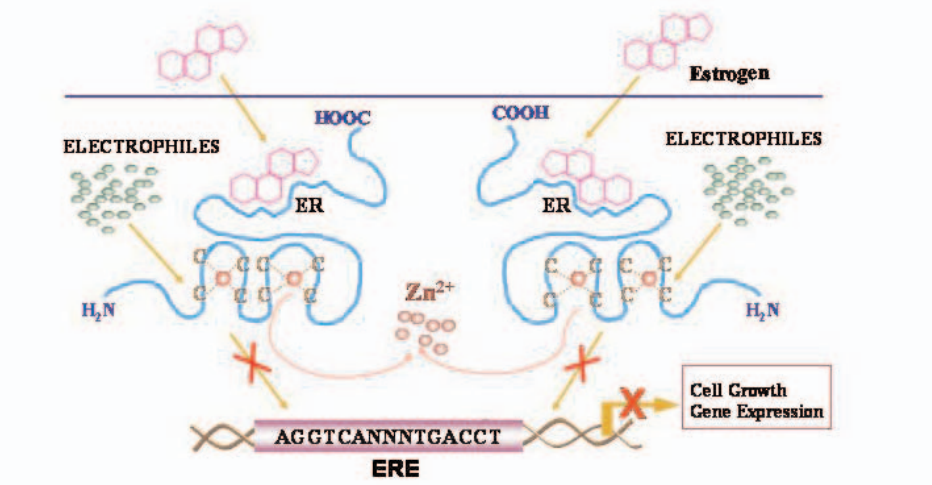

Figure 1. The estrogen receptor (ER) DNA-binding domain contains two nonequivalent Cys_4 zinc finger motifs that are essential to estrogen receptor–mediated transcription. The function of these zinc fingers is susceptible to chemical inhibition by electrophilic disulfide benzamide and benzisothiazolone derivatives, which selectively block binding of the estrogen receptor to its responsive element (ERE) and subsequent transcription. Consequently, these electrophiles inhibit estrogen-stimulated breast cancer cell growth and interfere with cell cycle and apoptosis regulatory gene expression.

estrogen receptor–DNA complex. Thus, the disruption of estrogen receptor zinc fingers could have a critical impact on estrogen receptor–mediated transcription and subsequently breast cancer cell growth. Interestingly, the C-terminal estrogen receptor zinc finger in the dimerization motif is more flexible and less well structured than the N-terminal zinc finger. The Cys thiolates of this zinc finger were also characterized as particularly labile, suggesting that the action of electrophilic agents could chemically modulate estrogen receptor DBD function. Notably, it has been demonstrated that the retroviral nucleocapsid p7 (NCp7) protein of human immunodeficiency virus type 1 (HIV-1), which contains two Cys_3His zinc fingers, can be inhibited by a variety of electrophilic agents. However, little is known about the potential interaction of electrophilic agents with the estrogen receptor zinc fingers and their possible effect on breast cancer.

Can breast cancer be suppressed at the level of receptor-DNA binding as opposed to hormone-receptor binding? More specifically, can electrophilic chemical agents undermine the function of the estrogen receptor–DNA complex and thereby inhibit the growth of breast cancer cells? We demonstrated that the function of zinc fingers within the estrogen receptor–DNA binding domain is susceptible to chemical inhibition by two electrophilic compounds: a disulfide benzamide called DIBA and a benzisothiazolone derivative known as BITA, which selectively block the estrogen receptor from binding to its responsive element and subsequently inhibit transcription. Moreover, these compounds significantly inhibit estrogen-stimulated cell proliferation and markedly reduce tumor mass in nude mice bearing human MCF-7 breast cancer xenografts, as well as interfere with the cell cycle and apoptosis-regulatory gene expression. Functional assays and computational analysis support a molecular mechanism whereby electrophilic agents preferentially disrupt the vulnerable C-terminal zinc finger, thus suppressing estrogen receptor–mediated breast carcinoma progression (Figure 1).

Because all nuclear receptors contain dual Cys_4 zinc fingers within their DBDs, we also tested whether the compounds in our study affect other nuclear receptors binding to their responsive elements. With the TranSignal protein/DNA arrays, EMSA, and transfection assays, we have found that DIBA selectively downregulated E2-induced estrogen receptor interaction to DNA, but failed to influence endogenous activities of other tested nuclear receptors, such as peroxisome proliferator–activated receptor γ (PPAR-γ), vitamin D3 receptor (VDR), thyroid receptor (TR), retinoid X receptor (RXR), and glucocorticoid receptor in E2-treated MCF-7 cells. In addition, the specificity of DIBA was tested on other zinc-dependent proteins such as histone deacetylase (HDAC) and NF-κB, a key non–zinc finger transcriptional factor involved in the cell cycle progression in estrogen receptor–negative breast cancer cells. The results further confirmed that DIBA selectively inhibits estrogen receptor–mediated breast cancer cell growth via inhibition of the estrogen receptor zinc finger function.

What are the medical implications of these findings? These results support the idea that a novel strategy for inhibiting breast cancer is to target the level of DNA binding, rather than the level of ligand binding. These findings show that DIBA has anticancer activity, *in vitro* and *in vivo,* in estrogen-mediated breast carcinoma. The results also demonstrate that DIBA inhibits breast cancer cell growth by selectively blocking estrogen receptor zinc finger function, without significantly affecting other nuclear receptors. These active compounds (disulfide benzamides and benzisothiazolone derivatives) constitute promising lead compounds in the treatment of breast cancer. Future research will focus on optimizing the selectivity and potency of these compounds in the treatment of breast cancer and determining whether they complement existing antiestrogen therapy. Moreover, targeting the molecular determinants that affect estrogen receptor transcriptional activity may have beneficial effects on tamoxifen-resistant tumors.

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Binding of Steroid Receptors to Regulatory Elements Is Transient and Periodic

Nagaich AK, Walker DA, Wolford R, and Hager GL. Rapid periodic binding and displacement of the glucocorticoid receptor during chromatin remodeling. *Mol Cell* 14: 163–74, 2004.

It is well established that nuclear receptors induce extensive modifications to the chromatin template during gene activation and repression, and much literature has been published describing many of these modifications. The alterations include primary sequence tags (acetylation, phosphorylation, methylation, sumoylation) on histone tails and other chromatin-bound proteins, as well as the rearrangement of nucleosome structures (often referred to as nucleosome mobilization). Less is known, however, about the actual structural changes to local promoter chromatin, and how these changes affect gene expression. To explore and understand the molecular events involved in this chromatin transition, we developed an *in vitro* system that accurately recapitulates the hormone-dependent transition in chromatin structure at the mouse mammary tumor virus (MMTV) promoter (Fletcher TM et al. *Mol Cell Biol* 20: 6466–75, 2000). The results from our studies presented a logical conundrum.

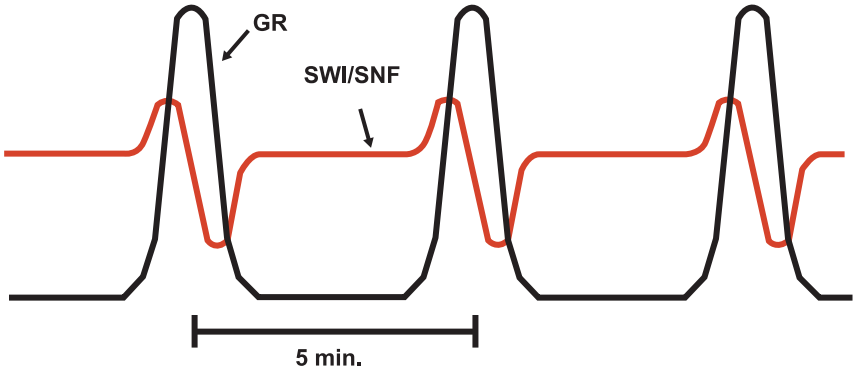

Figure 1. Periodic binding of transcription factors. The profile of laser-induced crosslinking during a 15 min *in vitro* chromatin-remodeling reaction is presented schematically for glucocorticoid receptor (GR) (black) and the SWI/SNF remodeling protein complex (orange). Each complex manifests a transient binding and displacement phase, followed by similar, repetitive events.

Although the hormone-induced transition was clearly receptor dependent, the receptor appeared to be lost from the template during the remodeling process. These findings led us to postulate a "hit-and-run" mechanism for receptor action (Fletcher TM et al. *Mol Cell Biol* 22: 3255–63, 2002) and also prompted us to recall our earlier findings from an *in vivo* study that showed the receptor residence time on the promoter to be quite brief (McNally JG et al. *Science* 287: 1262–65, 2000).

We discovered that classic assays for chromatin transitions (DNase I access, restriction enzyme access, methidium-propyl-EDTA [MPE] chemical cleavage) were inadequate to monitor receptor-induced events. Therefore, we decided to use a new approach involving laser UV light to follow the events in real time. Von Hippel and colleagues developed laser UV crosslinking to study protein/DNA interactions (Hockensmith JW et al. *J Biol Chem* 268: 15721–30). A laser UV light source has several advantages over conventional low-intensity light

New Required Intramural Acknowledgement in Publications Will Aid in Tracking of Intramural Contribution to Science

As of July 23, 2005, The NIH Office of Intramural Research requires that all intramural scientists include in the acknowledgements section of all of their publications the following statement: **"This research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research."**

The wording should be precisely as stated since it will be used to track the publications. Any divergence from this wording will hinder the tracking and lead to possible exclusion of the publication. The [in part] should be removed when the research was fully funded by intramural research.

NIH intramural research makes a large contribution to the world of science. However, that contribution is not always acknowledged or even known, while extramural contributions are, and have always been, carefully tracked.

Therefore, the reasons for this new requirement are two-fold: First, it will allow for tracking which publications come from intramural scientists despite variation in the way journals report addresses of co-authors. Second, this acknowledgement will highlight the important role that the intramural program plays in a great variety of innovative and collaborative research. As you know, scientists supported with extramural funds already are required to acknowledge NIH in their publications. Any questions should be directed to Tracy Thompson (thompstr@mail.nih.gov).

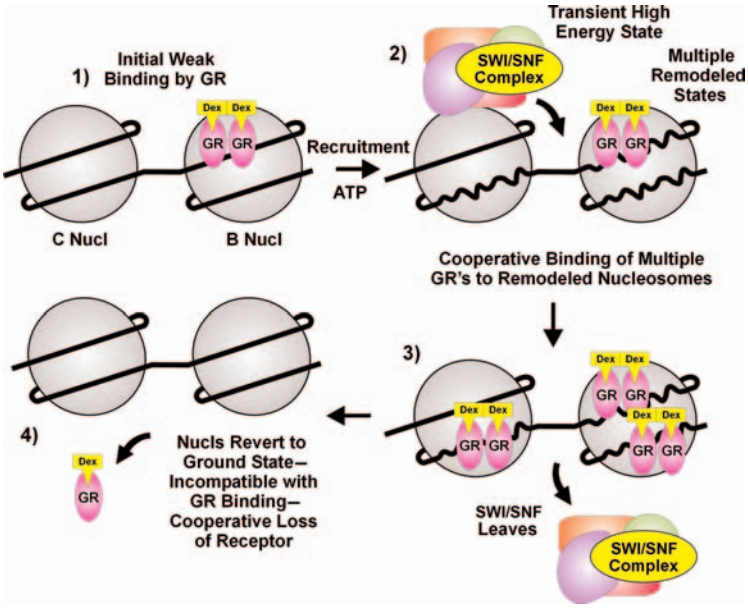

Figure 2. Model for the transient, periodic binding behavior of GR and SWI/SNF. C Nucl, C nucleosome; B Nucl, B nucleosome.

sources. For example, the high photon flux delivered by the laser generates radical cations of nucleic acid bases via a biphotonic mechanism. The high quantum yield of laser-induced radical cations leads to an efficiency of crosslinking exceeding that obtained with conventional UV light sources by at least two orders of magnitude. Finally, the crosslinking reaction itself is completed in less than 1 μs, allowing an analysis of rapid dynamic changes in protein-DNA interactions.

When we applied this new approach to analyze the interaction of glucocorticoid receptor (GR) and the SWI/SNF protein complex with the template during chromatin remodeling, we had surprising results (Nagaich AK et al. *Sci STKE* 2004(256): PL13, 2004; Nagaich AK et al. *Mol Cell* 14: 163–74, 2004). We found GR interactions with the template during the remodeling process to be highly transient and periodic (Figure 1). We observed a sharp peak in laser-detected binding at 5 min after initiation of the reaction, followed by equally rapid loss of the receptor. This cycle repeated periodically, with a cycle time of 5 min. We observed a similar cycle of binding for the SWI/SNF complex, although the detailed binding profile was different. There appeared to be a loss of SWI/SNF interaction as GR binding increased, with a return to the basal level of interaction as GR left the template. Laser-detected interactions of core histones with the template were also periodic, but more complex (Nagaich AK et al. *Mol Cell* 14: 163–174, 2004). Histones H2A and H2B each manifested a sharp peak during interaction, but these transitions were out of phase with each other.

These findings have led us to propose the following model for GR and chromatin remodeling complex interaction with the template: We suggest that the rapid binding of GR results from the initial recruitment of the SWI/SNF complex (Figure 2). At this stage, nucleosome remodeling "opens" the structure and increases the number of available GR binding sites. (There are a total of six binding sites in the B/C nucleosome region [Fletcher TM et al. *Mol Cell Biol* 20: 6466–75, 2000]). We propose that this local perturbed chromatin state is transient, leading to subsequent loss of the remodeling complex (Figure 1). Progression of the remodeling process would lead in turn to a collapse of the high energy state and a return of the local chromatin domain to the ground state. As this state is incompatible with binding of multiple GR homodimers, GR would be rapidly lost.

The implications of these findings, if generalized to other members of the receptor superfamily and other transcription factors, are quite profound and are leading to a paradigm shift in the field. Since the elaboration of the general receptor/DNA regulatory element model 30 years ago, it has been a central tenet in endocrinology that hormone-activated receptors bind stably to their regulatory sites and nucleate the formation of large multiprotein complexes. In contrast, our findings indicate that the receptor only briefly resides on the template. It appears that GR is actively ejected from the chromatin structure as a direct result of the progression of the remodeling process.

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The Center of Excellence in Immunology: A Record of Achievement and Leadership for the Future

Investigators at the CCR have been at the forefront of the paradigm shift illuminating the multifaceted relationship between the immune response and cancer. In the past 30 years, these research advances have begun opening the door to developing immune-based treatments for cancer and providing groundbreaking contributions in areas as diverse as cellular immunity, innate immunity, cytokines, and viral immunology. The translation of advances in basic research to the clinic has yielded a portfolio of immunotherapy research at the CCR that is unparalleled. Some bench-to-bedside accomplishments from the CCR, summarized in Table 1, include successful treatment of hairy cell leukemia using immunotoxins, radio-immunotherapy of refractory non-Hodgkin's lymphoma, and targeting the interleukin-2 (IL-2) receptor with monoclonal antibodies to treat T-cell leukemia, autoimmune disease, and graft-versus-host disease (GVHD). An exciting recent development is a cell-based therapy for the treatment of refractory metastatic melanoma that has resulted in improvement in 51% of patients involved in clinical trials. Given the bleak prognosis for those with late stage melanoma, these are remarkable and promising results.

Table 1. Selected Bench-to-Bedside Accomplishments of CCR Scientists in Developing Immunologic Approaches to the Prevention and Treatment of Cancer

- Basic, Translational, and Clinical Research on Human Papillomavirus (HPV), Leading to Phase III Clinical Trials of a Vaccine to Prevent Cervical Cancer.
- Cellular Therapy of Cancer, including:
  - Novel immunotherapy for refractory metastatic melanoma with a 51% response rate
  - Interleukin-2 (IL-2) in the treatment of metastatic renal cancer and melanoma
  - Advances in bone marrow transplantation
  - First clinical trials of IL-7 in humans
- Design and Use of Immunotoxins to Treat Cancer:
  - U.S. Food and Drug Administration (FDA)–approved treatment for hairy cell leukemia
  - Phase III clinical trials to treat brain, stomach, colon, and breast cancer
- Radio-immunotherapy of Cancer:
  - Facilitated development of Zevalin, a treatment for refractory non-Hodgkin's lymphoma (NHL)
  - Developed anti-CD25 to treat T-cell leukemia, autoimmune disease, and graft-versus-host disease (GVHD)
- Advances in the Design of Therapeutic Cancer Vaccines, including:
  - Identification of novel tumor antigens
  - Improvements in vaccine design
  - Treatment strategies that combine vaccines and conventional therapy
  - Generation and distribution of clinical-grade vaccines nationwide

The CCR is also host to several strong programs aimed at developing cancer vaccines. Basic research into the assembly of human papillomavirus (HPV) has been translated into a vaccine designed to prevent infection by this virus. This vaccine, currently in phase III clinical trials, has the potential to eliminate up to 150,000 deaths a year from cervical cancer. Further, therapeutic cancer vaccines from the NCI are in clinical trials throughout the nation. The unique blending of expertise in basic, translational, and clinical research and the ability of the NCI Intramural Research Program (IRP) to fund long-term, high-risk research have been key in developing each of these approaches to immunotherapy for cancer.

The Center of Excellence in Immunology (CEI), composed of a 19-member steering committee and a faculty of approximately 250, was formed to capitalize on the strength of the immunology community at the CCR. This umbrella organization cuts across and is inclusive of many existing Laboratory/Program/Branch structures to promote information exchange and collaborations among immunologists in the CCR. It also generates a multidisciplinary venue to further discovery, development, and delivery of novel immunologic approaches for the prevention and treatment of cancer. The steering committee meets monthly to discuss initiatives of potential benefit to the immunology community at the NCI, as well as means to address challenges to developing tools for immunotherapy. The 19 scientists on the CEI steering committee represent a continuum of expertise from basic to clinical research, having collectively published nearly 5,000 peer-reviewed articles since 1990. Further, the CEI faculty includes two members of the National Academy of Sciences and five members of the Institute of Medicine of the National Academy of Sciences. Thus, the CEI is uniquely suited to catalyze advances in basic, translational, and clinical immunology and to use this information to facilitate the development of successful immunotherapy for cancer.

One example of a CEI initiative is sponsorship of a National Immunotherapy meeting to facilitate information exchange and foster collaborations among NCI investigators, as well as other NIH scientists and the extramural research community. The meeting, organized by Dr. Jeff Schlom, is being held in the Masur auditorium on September 22–23. More information on this meeting can be found at http://web.ncifcrf.gov/events/tirc/. Persons interested in learning more about the current activities and future plans for the CEI are encouraged to attend a meeting of the CEI steering committee, held the third Monday of each month from 3–5 PM. Additional information on the CEI can be found at http://home.ccr.cancer.gov/coe/immunology/.

Work by scientists within the CEI has shown that immunotherapy can be extraordinarily effective in the treatment of a variety of cancers. Fueled by strong basic research programs and the ability to rapidly translate important findings to the clinic, investigators in the CEI are extending these findings, as well as developing additional tools to contribute to the NCI mission of eliminating death and suffering from cancer.

■ **Robert H. Wiltrout, PhD**
Director

■ MOLECULAR BIOLOGY

HMGA1: A Target of Dominant-Negative AP-1 When It Suppresses Transformation

Dhar A, Hu J, Reeves R, Resar LM, and Colburn NH. Dominant-negative c-Jun (TAM67) target genes: HMGA1 is required for tumor promoter-induced transformation. *Oncogene* 23: 4466–76, 2004.

Downregulating oncogenic transcription factors without affecting their normal function is a strategy that holds promise for cancer treatment and prevention. A promising target is the transcription factor activator protein-1 (AP-1). AP-1 plays a central role in neoplastic transformation and tumor progression in multiple organs. Suppressing elevated AP-1 activity as well as its upstream activators or downstream targets inhibits carcinogenesis in experimental models.

The AP-1 transcription factor consists of dimers of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2) proteins. Dimerization leads to their binding to gene promoters. Phosphorylation of the transactivation domain in response to extracellular stimuli (e.g., growth factors, cytokines, stress, and ultraviolet radiation) stimulates transcription of downstream genes. Although a basal level of AP-1 activity is required for normal cellular function, elevated levels of AP-1 activity result in cancer. AP-1 target genes responsible for tumor invasion or metastasis have been defined. However, AP-1 targets that are critical for transformation of a normal cell into a cancerous one have not been identified. Identification of such targets will allow better understanding of tumor induction and provide candidates for early stage preventive intervention.

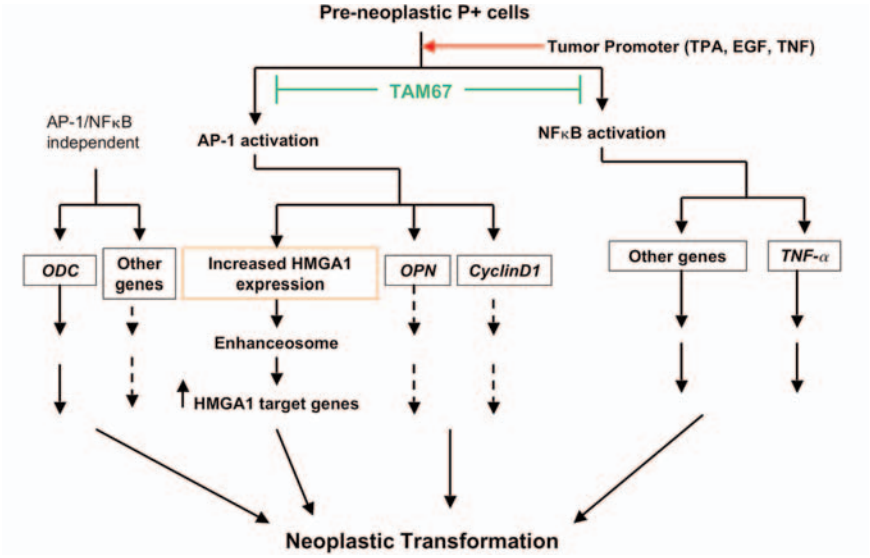

Figure 1. Dominant-negative AP-1 inhibits transformation by disrupting expression of high-mobility group protein A1 (HMGA1) and certain genes that cooperate to produce neoplastic transformation. TPA, phorbol 12-tetradecanoyl-13-acetate; EGF, epidermal growth factor; TNF, tumor necrosis factor.

We have used a dominant-negative c-Jun (TAM67) to identify AP-1–regulated genes that play a critical role in the neoplastic transformation response. TAM67 is a transactivation domain deletion mutant of c-Jun that retains the ability to dimerize with other AP-1 proteins. As a result, it acts as a dominant-negative suppressor of AP-1–dependent transcription, although it does not block all AP-1 activity. We have demonstrated that TAM67 blocks tumor promoter–induced transformation in mouse JB6 epidermal cells. The tumor promoters include phorbol 12-tetradecanoyl-13-acetate (TPA), tumor necrosis factor α (TNF-α) and epidermal growth factor (EGF). We also showed that TAM67 blocked tumor promotion and progression in a two-stage mouse skin carcinogenesis model. TAM67 does not block growth,

differentiation, or TPA-induced transient responses such as inflammation or hyperplasia in mouse skin. This indicates that TAM67 specifically blocks tumor induction-related events associated with excessive AP-1 induction, but does not block basal AP-1 activity required for normal cell function (Young MR et al. *Proc Natl Acad Sci U S A* 96: 9827–32, 1999).

We hypothesized that TAM67 inhibits the expression of a relatively small number of AP-1–regulated genes that are functional in tumor promoter–induced transformation. Our recent work shows that TAM67 inhibits expression of a small subset of the TPA-induced genes in mouse epidermal cells, while concurrently inhibiting AP-1 transactivation function. Furthermore, expression of two of the encoded proteins—c-Jun and high-mobility group chromatin architectural protein A1 (HMGA1)—was inducible by TPA and blocked by TAM67. Direct regulation of HMGA1 by AP-1 was confirmed by AP-1 protein binding to the HMGA1 promoter. Thus, expression of HMGA1 is regulated by AP-1 and blocked by TAM67 when TAM67 inhibits transformation. Suppression of HMGA1 protein expression by antisense-HMGA1 resulted in inhibition of tumor promoter–induced transformation of mouse skin cells, showing that HMGA1 is necessary for their transformation. Overexpression of HMGA1 was, however, not sufficient to confer the transformation-sensitive phenotype on cells deficient in critical tumor promoter–induced signaling pathways. This demonstrates that whereas HMGA1 is necessary for tumor promoter–induced transformation, it is not sufficient to confer the transformation-sensitive phenotype on mouse epidermal cells. (See Figure 1 illustrating the small number of genes whose elevated expression is critical to tumorigenesis, of which AP-1–regulated HMGA1 is one.)

Using the dominant-negative c-Jun, TAM67, we have identified an AP-1–regulated gene that plays a specific role in early neoplastic transformation. Although a number of AP-1 target genes, including *HMGA1,* are known to play a role in tumor *progression, HMGA1* is the first to be validated for its role in transformation of a cell in response to tumor-*inducing* stimuli. This further confirms the emerging concept that some molecular targets are common to tumor induction and tumor progression. Validation of genes important in tumor promotion genes provides targets for developing new and specific cancer prevention strategies. Use of TAM67, or a natural or synthetic molecule mimicking its effect, presents a promising strategy for cancer prevention. Furthermore, targeting functionally significant TAM67-downregulated genes such as HMGA1 may prove to be even more effective in preventing cancer. Basic and translational research efforts investigating these possibilities are under way.

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MOLECULAR BIOLOGY

Putting Plasmids in Their Places

Rodionov O and Yarmolinsky M. Plasmid partitioning and the spreading of P1 partition protein ParB. *Mol Microbiol* 52: 1215–23, 2004.

The ability of cells to make precise replicas of themselves is essential to the growth of every animal, plant, and microbe. To ensure that daughter cells receive a full genetic dowry, the duplicated genetic material must be appropriately partitioned between them. This orderly process of mitosis was first described in animal and plant cells more than a century ago, but the chromosomal ballet in bacteria is performed on such a small stage that, even with fluorescent designer tutus, the *pas de deux* is difficult to discern. Until recently, the bacterial equivalent of mitosis (binary fission) has resisted analysis of any kind. Our laboratory is addressing this challenging problem.

The approach we take is to study genetic elements still smaller than bacterial chromosomes: bacterial plasmids. For study, plasmids have two advantages over chromosomes: they are dispensable (do not code for any vital cellular structures or functions) and generally are equipped with no more than one mechanism for their partition to daughter cells. P1 is a plasmid of low copy number per bacterial cell and relies on specialized genes for partitioning. Homologs of these genes can be found in many bacterial chromosomes. The P1 partition system consists of two proteins (ParA and ParB) and a DNA site (*parS*)—the equivalent of a eukaryotic centromere—from which the plasmid is pushed or pulled. ParB binds directly to *parS*; ParA, an ATPase, can interact with the ParB-*parS* complex.

While trying to reconstitute the partition system of P1 from its separated parts, we encountered a surprising result. A small plasmid vector that was tagged with a drug-resistance marker and that carried the P1 centromere was *destabilized* by provision of a source of both Par proteins or of ParB alone. Drug-sensitive progeny bacteria were rapidly generated. Further work revealed that ParB silences expression of the drug-resistance gene and of a plasmid maintenance gene located close to *parS*. With *parS* in a more stable context—inserted within the bacterial chromosome—the silencing effect of ParB could be studied more readily. ParB was able to reduce the expression of

genes at least 5,000 base pairs away from *parS*. By cross-linking the DNA to protein and using antibodies to separate DNA specifically bound to ParB, we could show that ParB was capable of spreading from *parS* along extensive regions of flanking DNA.

The spreading phenomenon was unprecedented in bacteria, but what had it to do with the mechanism of partitioning? Evidence for its relevance to partitioning came from comparative and genetic studies. The capacity to polymerize from a specific binding site appeared to be a conserved property of some ParB homologs. Moreover, several mutant ParB proteins that were unable to destabilize the tester plasmid of our original experiments and that exhibited no obvious defect in binding to *parS* were found to be defective with regard to partitioning. Subsequent studies showed that each of these "spreading-negative" ParB mutants exhibited defects in promoting a regulatory interaction with the partition ATPase and also in pairing *parS* loci, an action generally accepted as a necessary first step in partitioning. These multiple defects cast serious doubts on the existence of a specific connection between ParB spreading and partitioning—the most compelling evidence for such a connection had become suspect.

We have examined the relevance of ParB spreading to partitioning. We used physical roadblocks flanking *parS* to confine the extent of spreading in a partitionable plasmid. Only when the roadblocks were within less than 200 base pairs from *parS* was partitioning diminished; the effect was slight. Why, then, should the unusual potential of ParB proteins to spread be conserved? Although we cannot answer this intriguing question with any certainty, one possibility is that the spreading of ParB provides a pool of the protein adjacent to its site of action on the plasmid, a pool that may hasten the pairing of plasmids when haste becomes important. And when might that be? Perhaps only in a competitive situation when an alien plasmid arrives that has a similar centromere and that could form mixed pairs capable of compromising partition of the resident plasmid. It may be that spreading is conserved because plasmids, like the rest of us, live in a highly competitive environment requiring investment in defensive measures. Recent experiments on plasmid competition by our "competitors" support this conjecture.

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TUMOR BIOLOGY

Hypoxia-inducible Factor—A Novel Target for Cancer Therapy

Palayoor ST, Burgos MA, Shoaibi A, Tofilon PJ, and Coleman CN. Effect of radiation and ibuprofen on normoxic renal carcinoma cells overexpressing hypoxia-inducible factors by loss of von Hippel-Lindau tumor suppressor gene function. *Clin Cancer Res* 10: 4158–64, 2004.

As tumors expand, the existing vasculature is unable to supply sufficient nutrition and oxygen to the rapidly proliferating cells. Tumor cells that are diffusion limited (approximately 100–150 μm away from blood vessels) and those that are perfusion limited (as a result of intermittent blood supply caused by abnormal tumor vasculature) are particularly affected, resulting in heterogeneous hypoxic areas in solid tumors. Although normal tissues typically have median oxygen concentrations in the range of 40 to 60 mm Hg, half of solid tumors have median values less than 10 mm Hg.

Like other eukaryotic cells, tumor cells respond to changes in oxygen levels through hypoxia-inducible factor (HIF), of the basic helix-loop-helix-PAS family of transcription factors. HIF is a heterodimer consisting of two subunits, α and β, the former being the oxygen-sensing subunit of HIF. Under normoxia, HIF-1α interacts with tumor suppressor von Hippel-Lindau protein (VHL), a component of the multiprotein ubiquitin ligase complex (Figure 1, part A). This results in ubiquitination and rapid degradation of HIF-1α via the proteasomal pathway. Under low oxygen conditions HIF/VHL interaction does not occur, allowing HIF-1α to escape ubiquitination and degradation. HIF-1α then dimerizes with HIF-1β, enters the nucleus, and activates target genes involved in erythropoiesis, glycolysis, and angiogenesis. In addition, HIF-1α enhances the expression of genes coding for growth factors/receptors, the apoptotic pathway, cell cycle regulators, and invasiveness. As a consequence of hypoxia or certain genetic alterations, the majority of human tumors overexpress HIF-1α and HIF-2α compared with surrounding normal tissue.

Clinical studies have shown that tumor hypoxia is not only a poor prognostic marker but also a major limiting factor for radiation therapy and some chemotherapeutic agents. Radiobiological studies have demonstrated that the biological effect of radiation is greatly influenced by the oxygen concentration at the time of irradiation. Hypoxic tumor cells are 2- to 3-fold more resistant to radiation than are well-oxygenated cells. Oxygen molecules rapidly react with the DNA damage produced by free radicals generated during ionizing radiation, making the damage permanent and irreparable, which then results in cell death.

Although hypoxic tumor cells are relatively resistant to radiation therapy, the precise role of HIF in radioresistance

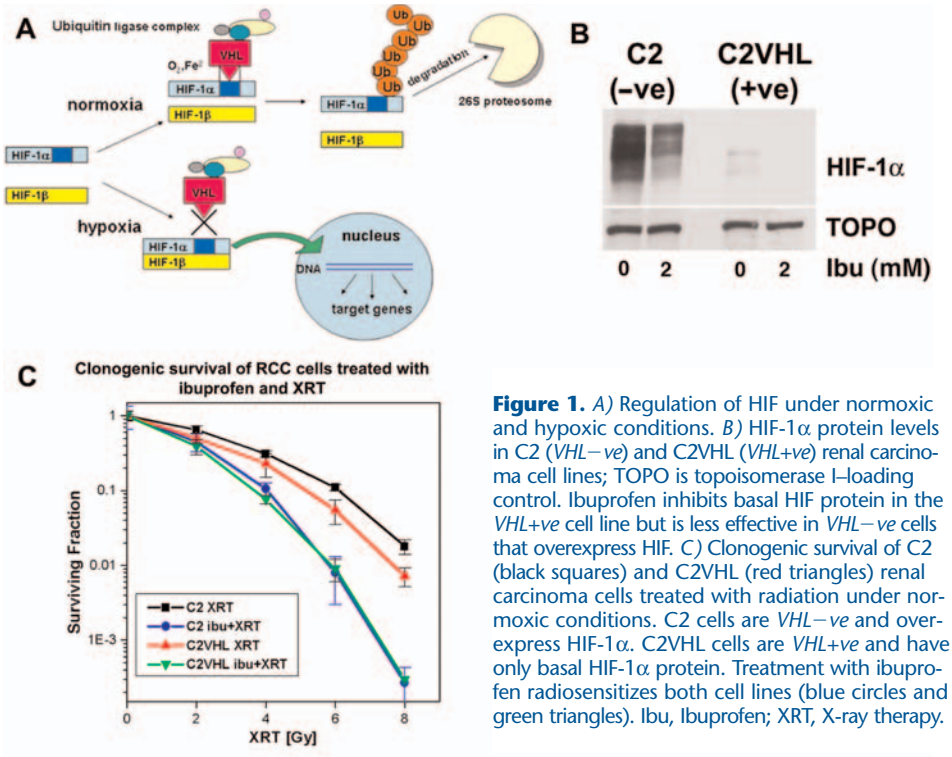

Figure 1. *A)* Regulation of HIF under normoxic and hypoxic conditions. *B)* HIF-1α protein levels in C2 (*VHL−ve*) and C2VHL (*VHL+ve*) renal carcinoma cell lines; TOPO is topoisomerase I–loading control. Ibuprofen inhibits basal HIF protein in the *VHL+ve* cell line but is less effective in *VHL−ve* cells that overexpress HIF. *C)* Clonogenic survival of C2 (black squares) and C2VHL (red triangles) renal carcinoma cells treated with radiation under normoxic conditions. C2 cells are *VHL−ve* and overexpress HIF-1α. C2VHL cells are *VHL+ve* and have only basal HIF-1α protein. Treatment with ibuprofen radiosensitizes both cell lines (blue circles and green triangles). Ibu, Ibuprofen; XRT, X-ray therapy.

is not established. Overexpression of functional HIF-1α or HIF-2α is commonly observed in hereditary and sporadic renal cell carcinomas (RCC) due to inactivation of *VHL* tumor suppressor gene (*VHL−ve* cells) (Figure 1, part B). Reintroduction of functional wild-type *VHL* gene in these RCC cells (*VHL+ve* cells) abrogates the overexpression of HIFs and HIF-regulated gene products. To evaluate the specific role of HIF-1α in the cellular radiation response, we irradiated matched *VHL−ve* (C2) and *VHL+ve* (C2VHL) RCC-paired cell lines under identical normoxic conditions. Despite the large differences in HIF-1α levels, there was only a modest difference in the radiosensitivity of the matched pairs (Figure 1, part C). Next, RCC cells were treated with the anti-inflammatory agent ibuprofen, a known radiosensitizer and HIF inhibitor of prostate cancer cells. Ibuprofen completely inhibited basal HIF-1α expression in *VHL+ve* cells but showed only a marginal effect on the overexpressed HIF in *VHL−ve* cells. Nevertheless, ibuprofen radiosensitized both cell lines to the same extent, suggesting that the radiosensitization by ibuprofen was not affected by HIF levels.

Our data on the normoxic HIF-overexpressing RCC cells suggests that in the setting of *VHL* mutation, HIF may not be the primary target for radiation *in vitro*. Interestingly, radiation upregulates proangiogenic cytokines like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which protect tumor vasculature and thereby could contribute to poor treatment outcome. Newer approaches to cancer treatment therefore involve targeting tumor cells as well as tumor vasculature and stroma. Inhibition of angiogenesis by inhibition of HIF is an attractive strategy that needs to be further explored. Novel therapeutic agents that inhibit HIF-1α activity include inhibitors of HIF-activating signal transduction pathways as well as inhibitors of important cellular targets like HSP90, topoisomerase I, and thioredoxin 1. The role of HIF inhibition per se using these inhibitors as potential anticancer agents remains to be determined. That HIF is selectively overexpressed in human tumors and, in turn, activates genes for tumor survival, growth, and metastasis makes it a novel target for combined modality treatment including radiation therapy.

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■ CANCER AND CELL BIOLOGY

The ABCs of Drug Resistance

Szakacs G, Annereau JP, Lababidi S, Shankavaram U, Arciello A, Bussey KJ, Reinhold W, Guo Y, Kruh GD, Reimers M, Weinstein JN, and Gottesman MM. Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. *Cancer Cell* 6: 129–37, 2004.

Chemotherapy is still one of the most effective ways to treat disseminated cancer. Unfortunately, even as multiple agents are used simultaneously, the effectiveness of chemotherapy is limited by multidrug resistance (MDR) of cancer cells. MDR affects many anticancer drugs, including hydrophobic natural products (e.g., doxorubicin and paclitaxel), new hydrophobic synthetic agents (e.g., imatinib mesylate), and more water-soluble drugs, such as cisplatin and methotrexate. "Classical MDR" is frequently associated with the decreased cellular accumulation of anticancer drugs and the elevated expression

of ATP-dependent drug-efflux pumps that belong to the superfamily of ATP-binding cassette (ABC) transporters.

ABC transporters are integral membrane proteins that transport a wide variety of substrates across cell membranes. Substrates include metabolic products, lipids, sterols, and xenobiotics such as chemotherapeutic drugs. There are 48 ABC proteins encoded in the human genome, grouped into 7 subfamilies ranging from A to G. Of these, only approximately 10 have been associated with cancer drug resistance, including the archetypal multidrug transporter ABCB1 (also known as MDR1 and P-glycoprotein), ABCC1 (MRP1), and ABCG2 (MXR). In addition, the high degree of sequence similarity between these ABC transporters suggested that further members of the A, B, C, and G subfamilies may also contribute to selected cases of multidrug resistance.

At present, relatively little is known about the substrate specificities of most ABC transporters. To determine which ABC transporters may be involved in drug resistance of cancer cells, we characterized the expression profile of the 48 ABC transporters in the National Cancer Institute 60 (NCI-60) cancer cell panel. The NCI-60 cell panel was set up by the Developmental Therapeutics Program (DTP) of the NCI in 1990, and more than 100,000 chemical compounds have been screened since then. Included among the 60 cells are leukemias, melanomas, and cancers of ovarian, breast, prostate, lung, renal, colon, and central nervous system origin. We hypothesized that if we were to measure the expression of ABC transporters, it would be possible to link ABC transport function to a variety of other already determined molecular, physiological, and pharmacological features of the cells.

We wished to focus our research, in particular, on the relationship between ABC expression levels and sensitivity to drugs or drug candidates, asking which of the transporters do (and which do not) confer resistance to various classes of agents. Since reproducible, quantitative correlations between expression and sensitivity were required for our study, we chose to measure transcript expression by the "gold-standard" method, quantitative real-time reverse transcription (RT)-PCR, rather than the less sensitive, less specific microarray technology. The RT-PCR data suggested that human cancer cell lines of various kinds express most of the 48 known ABC proteins. With help from collaborators in the NCI laboratory of John Weinstein, MD, PhD, we next searched for statistical correlations between the cell lines' sensitivity to cancer drugs and the expression of ABC transporters. Using this pharmacogenomic approach, we obtained precise correlations between ABC transporter expression and known patterns of drug activity for 1,429 compounds across the 60 cancer cell lines. Analysis of the resulting 68,592 ABC gene and drug relationships yielded 131 strongly inverse-correlated pairs, where the expression of an ABC transporter was strongly correlated with decreased sensitivity to a drug. As expected, good agreement was found between expression of MDR1 and reduced cellular sensitivity to anticancer drugs that are known to be substrates for this transporter. Furthermore, the method also allowed the identification of previously unknown MDR1 substrates.

To verify whether the highly significant negative correlations indicate functional relationships, in which an ABC transporter other than MDR1 protects the cells by exporting the related compounds, we performed independent follow-up experiments to compare control cells with transfected derivatives overexpressing various ABC transporters. In all tested cases, the follow-up experiments validated our predictions. As a result, several transporters that were not previously known to cause drug resistance were identified, suggesting that the real-time RT-PCR database and the analytical approach described above provide an unbiased method for discovering the substrate specificities of known, as well as yet uncharacterized members of the ABC superfamily.

Despite promising *in vitro* results, successful modulation of clinical MDR through the chemical inhibition of drug efflux from cancer cells remains elusive. Over the years, several generations of MDR1 modulators have raised hopes only to fail in clinical trials. The negative results may be explained by several factors, such as the intrinsic toxicity of the modulators and the unwelcome inhibition of MDR1 located in pharmacological barriers, resulting in the altered distribution of the simultaneously administered chemotherapy. Surprisingly, expression of some ABC transporters, most notably MDR1, caused an increase in the sensitivity of cancer cells to some drugs. This increase was unexpected, as MDR1 is perhaps the best-known multidrug resistance protein. A central hypothesis arising from our work is that these MDR1-inverse compounds, whose toxicity is potentiated rather than antagonized by the MDR1 multidrug transporter, might prove useful in patients whose tumors have already acquired resistance to chemotherapy.

We expect that our results will serve as a starting point for research leading to novel therapies designed to either evade or exploit the action of ABC transporters. The database created as a result of our work will provide a means to identify transporters whose expression confers drug resistance, and compounds whose effects are antagonized, unaffected, or even potentiated by transporter expression. The database (available at http://discover.nci.nih.gov/ABC) will also be valuable for future data mining to aid ABC transporter research.

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